REPRODUCTION AND ADAPTATION IN EASTERN PACIFIC EELGRASS POPULATIONS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JOSHUA STEPHEN NEELY find it satisfactory and recommend that it be accepted.

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Chair

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REPRODUCTION AND ADAPTATION IN EASTERN PACIFIC EELGRASS POPULATIONS

Abstract

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Zostera marina meadows frequently overlap tidal habitats in which different environmental conditions are found. The amount of clonal or sexual reproduction may reflect the level of disturbance found in the habitat. Also, timing of anthesis or stigma receptivity by intertidal eelgrass to coincide with low tides may increase pollination efficiency. How is reproduction in *Z. marina* influenced by tidal activity? This thesis addresses this question from three different perspectives: the effect of physical habitat on intra-population structure; the effect of tidal flux on intertidal pollination dynamics; and evidence of varying reproductive strategies among local intertidal and subtidal habitats. Chapter one investigates the population genetics of eelgrass in a habitat with dissected physical structure. Findings indicate that little structure exists between discrete beds, and genetic diversity is not consistent with widespread clonality. This suggests efficient gene flow across channels, likely by many genets. Chapter two examines the influence of tides on the presentation and dispersal of eelgrass pollen. Results suggest that timing of

pollination is not coordinated with bi-monthly tidal events, and that a strategy relying on pollen-concentrating low tide would interfere with pollination. Chapter three investigates reproductive strategies of *Zostera marina* in nine populations. Results suggest that *Z. marina* reproductive strategy does not differ between habitats. Evidence of reproduction through sex and clonal growth were both found to be scarce. These studies can aid the management of eelgrass populations by discouraging the assumptions of low genetic diversity and little sexual reproduction. Intertidal habitats do not appear to closely follow many of the hypotheses generally applied to them.

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INTRODUCTION

Plant reproductive strategies represent adaptations to both biotic and abiotic components of the environment. Marine angiosperms face unique challenges during reproduction, and apparent adaptations characterize their mode of existence. Core concepts of clonality, geitonogamy, and hydrophilous pollination offer a framework for hypothesis formation. Many marine angiosperms engage in extensive clonal reproduction. Clonality allows modular specialization (e.g., rhizomes that provide anchorage) and replication in physiologically independent units. However, clonality becomes a component of the breeding system by making geitonogamy (between-flower self-fertilization) a likely outcome. Also, most marine angiosperms rely on hydrophily for sexual reproduction. Tidal action creates varying pollination environments: subsurface (submerged inflorescences); surface (floating inflorescences); and no pollination (emergent inflorescences). Some habitats are affected by tidal action more than others, and as a result may engage in different reproductive strategies. This dissertation presents three field experiments on the reproductive ecology of *Zostera marina* L., marine eelgrass.

Chapter one presents an investigation of intra-population structure and the effect of discrete habitat edges on it. The study finds that clonality is not widespread, with evidence of gene flow between habitats. The intertidal nature of the *Z. marina* population under investigation may limit the effects of habitat edges by adding a third physical dimension, during submersion, which encompasses all portions of the habitat, whether located at the edge or in the interior.

Chapter two considers the effect of low tide's approach on pollination dynamics. Does *Z. marina* time pollination to exploit this important aspect of the environment? Conditions around the time of low tide do not concentrate pollen, so much as limit its effective dispersal. Hypotheses on seagrass pollination that favor either surface or subsurface pollen dispersal are considered in light of the results. The likelihood of interference in pollen dispersal and the lack of coordination in flowering phenology during low tide suggest that *Z. marina* may be better suited to pollinate underwater.

Chapter three investigates fine-scale and broad geographic patterns of morphological and molecular differentiation, emphasizing the discrete habitats that differ qualitatively due to tidal action. Different hypotheses from the aquatic plant literature, contrasting assured clonal reproduction against genetic diversity through sex, are considered. Inter-population results from nine sites spanning over 1000 miles of species range are presented. Genetic variation is low between habitats, suggesting gene flow, and the plants of neither intertidal nor subtidal habitat show differing levels of clonal growth or production of reproductive structures. Reproductive strategies do not appear to reflect the existence of discrete intertidal and subtidal habitats.

CHAPTER ONE: EFFECTS OF PHYSICAL HABITAT ON THE POPULATION GENETICS OF HUMBOLDT BAY EELGRASS

Abstract

The physical structure of a habitat (e.g., extent of subdivision or of population 'edges') may affect the distribution of genetic diversity within a population, and genetic diversity may alter ecological function. This study investigates the population genetics of Humboldt Bay eelgrass in light of the habitat's dissected physical structure. A sample of 469 individuals, from 11 discrete beds, indicates that genetic diversity does not differ among beds, and is not consistent with widespread clonality. This suggests efficient gene flow across channels. Heterozygosity levels indicate that H-W equilibrium predominates across loci in most instances. These observations can aid the management of Humboldt Bay eelgrass by discouraging the assumptions of low genetic diversity and little sexual reproduction, which will aid in the formation of evolutionarily-informed conservation decisions.

Introduction

Zostera marina L. (Zosteraceae; marine eelgrass) relies on clonal growth and sexual reproduction (Den Hartog 1970; Sculthorpe 1967). Clonality in aquatic environments may be a response to the uncertainty of pollination, or a means to exploit stable environments (Les 1988). Clonal populations that lack effective sexual recruitment may still consist of more than just one genet (Ellstrand and Roose 1987) and the extent of clonal spread can be large (Barrett et al. 1993; Grace 1993). Sexual reproduction contributes to genetic diversity, and is known to influence the structure of *Z. marina* populations (Reusch 2000b). In bisexual species, the spatial aggregation of clones creates a pollination environment that encourages geitonogamy (Handel 1985).

Marine environments, especially intertidal seagrass beds (i.e., mudflats inhabited by eelgrass), differ from non-marine environments (Laushman 1993). Physical aspects of the marine environment (e.g., water as a propagule vector) may reduce typical avenues of gene flow (Les 1988; Orth et al. 1994; Ruckelshaus 1996), so that genetic structure may be easily established in seagrass populations occupying heavily-fragmented habitats. Physical fragmentation of beds also creates peripheral zones that may be subject to different population-shaping forces (e.g., mortality, gap formation, propagule immigration, etc.) than interior zones. This is because ramets on the edge of a habitat act as a buffer, reducing the force of water flow and mixing in the interior zone (Ackerman and Okubo 1993). As a result, edge habitat may receive more incoming reproductive propagules, and edge disturbances may cause more mortality and turnover of individuals. On the other hand, regular submersion of the entire bed during high tide events may

constrain the effects of such buffering. The genetic structure of seagrass populations is likely to reflect these disparate forces. Microsatellite analyses of seagrass populations have demonstrated widely-varying genetic diversity between populations (Procaccini et al. 2002; Reusch et al. 2000), but it is clear that populations are not the homogenous assemblages of sister ramets that they were perceived to be (Barrett et al. 1993; Reusch 2001b; Reusch et al. 1999b).

This study surveys the genetic structure of a population of *Z. marina* located in the eastern Pacific Ocean at Humboldt Bay, California. Measurements of genetic diversity may provide insight into processes structuring genetic variation. The physical structure of eelgrass habitat in the bay presents an ideal opportunity to study the genetic effect such structure can have within a population. Within a bed, we suggest that genetic diversity primarily represents clonal diversity. At larger scales, the barriers to clonal spread render genetic diversity more a measure of gene flow. Our first hypothesis is thus that genetic differentiation closely follows the physical structure (see *Study System*, below) of the habitat. To evaluate this hypothesis, we will use microsatellites to assess genetic diversity, heterozygosity, and molecular variance of the population at different spatial scales.

Limited propagule and clonal dispersal distances and population structure due to clonality suggest that the reproductive opportunities available at the edge of an eelgrass bed may differ from those available in the interior. Reduced fluid mixing in the interior may hamper reproductive function (Ackerman and Okubo 1993), especially outcrossing, by diminishing the size of an individual's pollen shadow and making it less likely that pollen disperses beyond the boundary of the genet. More generally, less water movement

would mean less pollen movement and hence less pollination, outcrossing or otherwise. For these reasons, our second hypothesis is that there is greater homozygosity and clonality in the interior compared to the periphery of *Z. marina* beds. To evaluate this hypothesis, we compare genetic diversity, heterozygosity, and molecular variance of edge and interior samples.

Methods

Study System

Zostera marina is a common seagrass throughout the northern hemisphere. It is typically found in sheltered estuarine embayments (Den Hartog 1970). Individuals of the species are monoecious, bearing imperfect flowers of both sexes together on long, flattened inflorescences. Flowers of *Z. marina* exhibit protogyny; female flowers conclude their phase of pollen receptivity at least 24 hours before the initiation of anther dehiscence (De Cock 1980). The species is self-compatible (Ruckelshaus 1995). *Zostera marina* populations can be either annual or perennial, depending on local environmental conditions. Annual populations are most likely encountered in relatively unsheltered habitats, or at the north latitudinal extreme of the species range, where harsh seasonal conditions, such as freezing, reduce the survival of eelgrass (Den Hartog 1970; Reusch 2000b). Perenniality is typically achieved through rhizomatous growth, since above-ground shoots tend to deteriorate over the course of a growing season in the near-shore habitat. The rhizomatous habit also makes clonal replication likely. *Z. marina* habitats vary, from shallow subtidal to intertidal, where brief, regular periods of exposure to a terrestrial environment occur.

Humboldt Bay is located on the north coast of California, USA, and supports one of the largest *Z. marina* populations in that state. Individuals at Humboldt Bay produce flowering shoots as well as rhizomes, suggesting that the local population is maintained by a combination of sexual reproduction and clonal growth. Eelgrass habitat in the interior of the bay is subdivided into distinct intertidal mudflats bounded by dredged channels that provide relatively little subtidal habitat. Habitat around the inner periphery of the bay consists of gradually-sloping beaches, providing intertidal and subtidal habitat. Dredging has shaped most of the eelgrass habitat into isolated mudflat beds with discrete edges that are exposed during low tides. Spread of a genet between these habitat sites is unlikely since there is no medium for rhizome growth present, and establishment of eelgrass from vegetative propagules has a low probability of success (Ewanchuk and Williams 1996).

Specimen Collection

Fresh plant material was collected at low-tide periods during June 2003. Sampling was conducted in eleven different eelgrass beds (Fig. 1). Five beds were sampled in the northern portion of the bay, while three beds were sampled in each of the central and southern portions. Sampling did not occur in any areas of restored eelgrass habitat. Sampling was conducted within one 20m x 40m plot per bed. The location of each plot was determined such that one narrow side was placed adjacent to the low tide boundary of the bed (the edge) while all other sides were at least 3 m distant from the edge of the bed (i.e., the beds in which the plots were placed were larger than the plots themselves). Each plot had five sampling points, two of which were placed at random intervals along

the bed edge and three located at random locations in the interior of the plot. At each sampling point, up to nine leaves were collected from different eelgrass ramets in a 1 m radius. The specimens were coated in silica gel crystals and then stored at -20˚ C.

Laboratory Procedure

Extraction of DNA for microsatellite analysis was conducted with the aid of a Promega Wizard Genomic DNA purification kit. One extraction was performed for each ramet collected. Extracted DNA in buffer solution was analyzed for DNA concentration using a spectrophotometer, then diluted to a standard of 50 ng/µl. These working samples were frozen at -20°C until needed for PCR.

Samples were prepared for PCR by combining reagents in a 96-well thermocycler plate for a Techne Genius thermocycler. Each well of a plate contained: 2.95 µl salt/buffer solution, 1.0 µl DNA sample, 0.5 µl of each forward and reverse primer to be used, and 0.05 µl Taq. The CT-12 and CT-20 primers were labeled with VIC and the GA-2 and GA-3 primers were labeled with NED fluorescent labels, to allow multiplexing of primers within wells. Microsatellite sequences were taken from published records (Reusch 2000a; Reusch et al. 1999b). Once a plate was prepared, it was immediately loaded into the thermocycler and processed using the following cycles, durations, and temperatures: one cycle at 94°C for 2 min, 40 cycles at 94°C for 15 sec/50°C for 15 sec/72°C for 30 sec, and one cycle at 72°C for 30 min. Upon completion of PCR, the plate was refrigerated at 5°C until the samples were needed for genotyping.

Samples of amplified DNA were prepared for genotyping by transferring a 0.5 µl sub-sample to a new 96-well plate where the wells had been loaded with a mixture of

10.5 µl formamide and 0.5 µl LIZ 500 size-standard. Once a plate was prepared for genotyping it was processed at an on-site sequencing facility, where it was stored at 5°C until it was analyzed by an ABI 3730 DNA analyzer that night.

Data Analysis

Data output from the sequencer was visualized using ABI Prism Genemapper 3.5 software. All genotypes were manually scored for base pair length. Once all individuals were genotyped, scored 'alleles' differing by a small number of base pairs were grouped into a single allelic class. This approach assumed some error in the level of accuracy of sequencing, since assignment of a unique allele to every base pair length reported would have resulted in an extremely high and unlikely number of unique alleles. Analysis was of the full data set (469 individuals), and of a "trimmed" data set (360 individuals) in which individuals lacking unequivocal multilocus genotypes were removed.

Two indices of clonal diversity were computed for each sampling plot, as well as for edge and interior samples. The first, which computes diversity as the number of genets divided by the number of ramets (Ellstrand and Roose 1987), has previously been used to compute clonal diversity in eelgrass populations (see Reusch et al. 2000). Because this method does not account for relative frequency of genotypes, Simpson's diversity index was also computed for the same data. Simpson's diversity index (Hangelbroek et al. 2002) computes the relative frequency of each genotype present, then sums the results for a final value indicating total diversity. One-way analyses of variance were used to compare diversity measures in the 11 beds included in the survey. Variance between positions within a bed (edge or interior) was assessed using a nested ANOVA in

which bed position was used as the main effect and each diversity index served as a response variable. Analyses were conducted using the JMP IN 5.1.2 software package (Sall et al. 2003).

Data were also analyzed for deviation from Hardy-Weinberg equilibrium (HWE) and analysis of molecular variance (AMOVA) using ARLEQUIN version 2.0 (Schneider et al. 2000). For the HWE analysis, the full data set was grouped into separate beds as well as edge and interior samples. These sample sets varied in the number of observations included in each. Computations for HWE used default settings (100,000 steps in Markov chain; 1000 de-memorization steps). To assure experiment-wise error rate of 0.05, Bonferroni-corrected comparison-wise alpha values were used to determine significant differences.

Two types of AMOVA were performed using sampling point data to compose ARLEQUIN samples. In the first AMOVA, the data set was organized for analysis of differentiation between plots. Data collected at each point were placed in separate ARLEQUIN samples, for a total of 54 samples. These were further grouped by plot. In the second AMOVA, the data set was organized for analysis of differentiation between bed positions. Both AMOVA used the trimmed data set, to prevent 'incomplete' data for entire loci from being discarded by the software. ARLEQUIN profiles used are presented in the Appendix.

Results

Genetic characterization

The microsatellite loci used in this study were all polymorphic with six to eight alleles each (Table 1), and showed a high amount of allelic diversity for an eastern-Pacific population of *Z. marina* (Olsen et al. 2004). For each locus, there were one or two alleles and genotypes that occurred much more frequently than the others.

Mean genetic diversity varied widely among plots and less so among bed positions (Table 2). Ellstrand and Roose's diversity index resulted in significantly different means ($p = 0.0248$) among plots. There were no significant differences for between bed positions for Ellstrand and Roose's diversity index, or for Simpson's diversity index at either grouping.

Comparisons of observed (H_O) and expected (H_E) heterozygosity showed few departures from HWE throughout the population (Table 3). Differences in the departure from HWE needed to produce a significant result most likely result from unequal sample sizes. Among loci, CT-20 showed higher heterozygosity than expected in three instances and a single instance of lower-than-expected heterozygosity. GA-2 showed two instances of lower-than-expected heterozygosity. Among sampling plots, S-1 and S-3 showed one locus with lower than expected heterozygosity. C-1 showed one locus with higher than expected heterozygosity. Among bed positions, edge samples showed one instance of higher-than-expected heterozygosity. Interior samples showed lower heterozygosity than expected in one instance (all significant experiment-wise $p < 0.05$).

Population Structure

Tables 4a and 4b present the full results of the AMOVA for each data set. In the data set in which samples were grouped by plot, most of the detectable variation (approx. 84%) occurs within sampling points (i.e., among the nine individuals sampled per point). Variation within plots is approx. 15% of the total. Variation between sampling plots accounted for less than one percent of the total.

The bed position data set shows that the amount of variation found within and between sampling points is very similar to the plot data set. Very little variation (0.52%) is detected between edge and interior regions.

Discussion

Analysis of four polymorphic loci in the eelgrass population at Humboldt Bay, CA, showed infrequent deviations from HWE, little difference in genetic diversity across the bay, and most genetic variation present at the finest scale analyzed. Here we explore the roles that microevolutionary forces such as non-random mating and gene flow play in this system. Also, we suggest that tidal cycles moderate differences that might otherwise occur between edge and interior positions in a bed.

The surprisingly high mean values of genetic diversity at the plot level of analysis suggest that the spread of clonal genets is limited. This reinforces conclusions (Reusch et al. 2000) that clonality is not as pervasive as once thought. Different genetic diversity indices do, however, disagree about diversity between plots. Relative frequency of genotypes varies widely in our data, with few common types and many scarce types. As a consequence, Simpson's diversity index (Hangelbroek et al. 2002) may be preferable to

Ellstrand and Roose's diversity index (1987). This is because Simpson's index accounts for relative genotype frequencies. Results of Simpson's diversity index, then, show that genetic diversity in Humboldt Bay does not differ among beds. Some physical variation exists around the bay (e.g., habitats found in the south part of the bay are marginally higher in elevation than those in the north part of the bay), but such differences do not appear to influence the extent of clonality in Humboldt Bay.

Three of the six instances of significant deviation from HWE were due to lower-than-expected values. Significant departures from expected values indicate a violation of one or more assumptions (Hartl and Clark 1997). The most likely explanation for lower-than-expected heterozygosity observed in this study is that departures result from non-random mating, i.e., that higher incidences of self-pollination result in more homozygotes. The clonal nature of eelgrass makes geitonogamous selfing likely at finer scales, where a ramet is usually in the presence of sister ramets, and the limited dispersal ability of sexual propagules makes escaping the spread of one's own genet uncertain (Orth et al. 1994; Reusch 2001a; Ruckelshaus 1996). Genetic drift, due to small population size, could also cause deviation from HWE in neutral loci. Although one *Z. marina* bed, taken alone, could constitute a suitably small population for genetic drift to play a role, it is unlikely in this population since results of AMOVA suggest effective gene flow between beds (Slatkin 1987). However, deviations from HWE were few and low observed values only accounted for one half of all deviations.

Factors contributing to excess heterozygotes are less clear, but may include gene flow from other populations (Laushman 1993). Plots occupying northern and central locations in the bay show a single instance of excess heterozygosity, but no instances

where it is lacking. Plot Central-1, which exhibits excess heterozygosity at one locus, is located near to the mouth of the bay where rafting shoots from other populations would have to pass through to enter this population. These results suggest that non-random mating (likely due to geitonogamy) and gene flow are occasionally effective forces in the Humboldt Bay eelgrass population. Evidence of non-random mating is seen in the southern portion of the bay, while all northern and central samples are in equilibrium or show excess heterozygotes. Still, deviations from HWE are uncommon across the population, mostly occurring in the southern portion of the bay and between bed positions (see below).

Analysis of molecular variance showed decreasing variability as scale increased. Most of the observed genetic variability occurred at points, the smallest replicated scale with a 1m radius. This suggests that the nearest neighbors are rarely clones. This may mean that most sampled individuals at that scale originated from seed, or genets have become highly interdigitated through growth. Variability occurring within plots (approx. 800 m^2) was less pronounced, suggesting that the cohort of sampled genotypes often overlapped between points. At that scale, large clones and short-range propagule dispersal may both contribute to homogenization of variation. Negligible variation was observed between plots; this result is perhaps most surprising, considering the presence of the many channels that act as barriers to new clonal growth between bed habitats. Two possible explanations exist to explain the extreme homogenization observed. First, propagule dispersal may be occurring in a regular fashion over the entire bay. At such a scale, rafting reproductive shoots may play a role in the projection of propagules beyond their limited ranges when moved singly (Reusch 2002). Also, old genets may persist

across beds because the channels that would currently prevent clonal spread between beds did not always exist. However, while genets have been described as potentially very long-lived (Eriksson 1993) and the time since the installation of most channels is only on the order of decades, trauma from channelization efforts and historical oyster harvesting activities would have eradicated many individuals belonging to the pre-channel genets.

Edge effect

Our results provide little evidence that reproductive strategies vary between edge and interior bed positions. No differences were found between edge and interior samples for genetic diversity (Table 2). Observed heterozygosity deviated from HWE at both bed locations, but deviations at the interior bed position are both above and below expected values (Table 3). In a situation of limited sample sizes contradictory results may be attributable to sampling error. However, sample sizes here seem adequate. The influence of selection is the most reasonable explanation why some loci would exhibit excess heterozygosity while another exhibits the opposite. The problem, in this case, is that our microsatellite loci are assumed to be neutral. The effect of the physical environment is uncertain; one conclusive result of excess heterozygosity at the edge, versus conflicting results in the interior, could be interpreted as evidence of greater gene flow impacting the edge. Alternatively, conflicting results for the interior may suggest, but weakly, that non-random mating and gene flow are each affecting the edge in similar measure (or, that selection is somehow influencing neutral loci). Genetic variation was similar to that of the plots, where variation at the largest scale (between bed positions) contributed very little to total variation. Since edges and interiors were frequently adjacent, clonal spread

of genets between positions could account for at least part of the homogenization observed at that scale.

The expected relationship between individuals occupying edge and interior positions may have been weakened by the environmental conditions that all individuals are subjected to during certain times of the tidal cycle. The bed habitats are submerged daily; during submersion events the addition of a third dimension creates a vast, uniform edge above the canopies of both bed positions. This would not undermine the observed physical differences between positions in a seagrass bed (Ackerman 2002; Ackerman and Okubo 1993), but it suggests other forces that may be constraining the selection of certain reproductive strategies in populations that are subjected to regular tidal variance.

Value of genetic diversity

Seagrass stands are typically limited to one or a few plant species (Duarte 2002), which could undermine their perceived conservation value when placed next to relatively species-rich habitats. Measures of genetic diversity offer an alternative for the assessment of conservation value in seagrass habitats. Two similar recent studies (Hughes and Stachowicz 2004; Reusch et al. 2005) empirically tested the role of genetic diversity in a seagrass population's ability to recover from disturbances. They find that the time needed for a population to recover is less with increasing genetic diversity, and that, in addition to increased production of seagrass biomass, increased abundance is also seen in other members of the community. The effects are explained by the overall complementary effect of the genotypes present, rather than selection of those genotypes most well-suited to the conditions of disturbance, in this case unusually high temperature

(Reusch et al. 2005). The results presented here may be useful to natural resource managers of Humboldt Bay after disturbance events such as high temperatures, herbivore activities, or disease, affect the eelgrass population located there. Specifically, variation in the recovery of eelgrass at different locations in the bay may be attributed to factors other than genetic diversity, since genetic diversity was not found to appreciably differ among bay locations.

Near-shore habitats face many forms of disturbance, anthropogenic or otherwise (Short and Wyllie-Echeverria 1996). The results of these other studies show how closely linked the communities occupying intertidal seagrass beds are to these plants. In light of their results, more of the world's habitat-providing seagrass populations should be evaluated for levels of genetic diversity, before mitigation and development interfere with them. Such knowledge will provide a reasonable metric for assigning ecological value and predicting recovery ability of communities that exhibit a lack of certain types of species.

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Tables

Table 1.1. Number of observed alleles, and frequencies of most common allele and genotype for each locus.

Locus	Number of alleles	Allele frequency	Genotype frequency
CT-12	8	0.767	0.595
CT-20	8	0.504	0.523
GA-2	6	0.610	0.407
GA-3	6	0.802	0.638

Table 1.2. Mean values ± standard errors of genetic diversity indices; by plot, bed position, and the entire population; p-values from tests of significance of genetic diversity. Significance at the level of $\alpha = 0.05$ is indicated by '*'.

Plot	Ellstrand & Roose	Simpson
S-1	0.67 ± 0.09	0.96 ± 0.07
S-2	0.58 ± 0.06	0.92 ± 0.03
S-3	0.67 ± 0.11	0.94 ± 0.14
C-1	0.79 ± 0.09	0.96 ± 0.04
C-2	0.57 ± 0.12	0.94 ± 0.09
C-3	0.82 ± 0.08	0.96 ± 0.09
N-1	0.34 ± 0.18	0.85 ± 0.20
N-2	0.64 ± 0.11	0.92 ± 0.09
N-3	0.47 ± 0.10	0.94 ± 0.07
N-4	0.83 ± 0.07	0.97 ± 0.03
N-5	0.44 ± 0.05	0.94 ± 0.11
Edge	0.63 ± 0.06	0.75 ± 0.06
Interior	0.62 ± 0.05	0.77 ± 0.04
Population	0.62 ± 0.04	0.76 ± 0.03

Plot	Ellstrand & Roose	Simpson
Tests of significance		
Among plots	0.0248*	0.1126
Among bed positions	0.8603	0.7175

Table 1.3. Values of observed and expected heterozygosity at four microsatellite loci. Data is arranged by plot and bed position. Significant p-values are denoted by '*' which indicates a departure from Hardy-Weinberg equilibrium. To assure experiment-wise error rate of 0.05, Bonferroni-corrected comparison-wise alpha values of (a) 0.00114 and (b) 0.00625 were used.

	Locus											
	CT12			CT20			GA2			GA3		
	Obs	Exp	Sig	Obs	Exp	Sig	Obs	Exp	Sig	Obs	Exp	Sig
a. Plot												
S-1	0.44	0.57		0.62	0.66		0.16	0.64	*	0.07	0.15	
S-2	0.14	0.16		0.68	0.53		0.61	0.50		0.44	0.45	
S-3	0.57	0.49		0.39	0.56	*	0.51	0.62		0.50	0.45	
C-1	0.65	0.50		0.84	0.51	*	0.22	0.23		0.49	0.50	
C-2	0.14	0.16		0.52	0.50		0.41	0.52		0.57	0.46	
C-3	0.29	0.26		0.54	0.51		0.44	0.52		0.38	0.35	
N-1	0.04	0.28		0.75	0.59		0.61	0.63		0.14	0.25	
N-2	0.43	0.52		0.70	0.56		0.43	0.48		0.34	0.37	
N-3	0.51	0.66		0.71	0.57		0.60	0.50		0.35	0.4	
N-4	0.31	0.44		0.75	0.58		0.71	0.60		0.34	0.33	
N-5	0.55	0.56		0.63	0.56		0.43	0.65		0.19	0.24	

	Locus											
	CT12			CT20			GA2			GA3		
	Obs	Exp	Sig	Obs	Exp	Sig	Obs	Exp	Sig	Obs	Exp	Sig
b. Bed position												
edge	0.30	0.35		0.64	0.55	*	0.48	0.53		0.29	0.33	
interior	0.43	0.50		0.65	0.58	*	0.44	0.58	*	0.37	0.42	

Table 1.4. AMOVA results for the plot and bed position data sets. The majority of the total genetic variation shown in both tables occurs at the finest scale included in the analysis ('points').

Source of variation	DF	Sum of squares	Variation components	Percent of variation
Among plots	10	16.63	0.004	0.83
Among points within plots	43	56.46	0.071	15.29
Within points	670	259.46	0.387	83.88
Total	723	332.54	0.462	
Among bed positions	1	2.339	0.00242	0.52
Among points within bed positions	52	70.746	0.07292	15.76
Within points	670	259.455	0.38725	83.71
Total	723	332.540	0.46258	

Figure

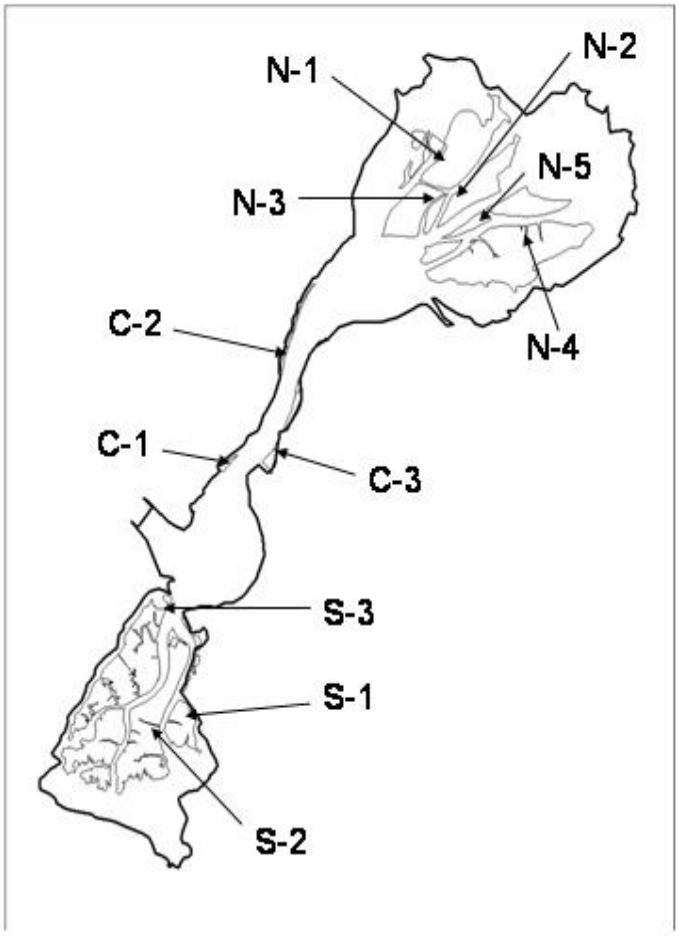

Figure 1.1. Map of Humboldt Bay showing plot locations. Eelgrass beds are outlined in gray.

CHAPTER TWO: TIDAL ROLE IN CONCENTRATING EELGRASS POLLEN

Abstract

There are two hypotheses about the nature of adaptation in eelgrass pollination. The first hypothesis is based on search theory and random motion, and the second on the mechanistic basis for submarine pollination. The former supposes that pollen search vehicles benefit from adaptations (e.g., in flowering phenology) resulting in pollen dispersal in *two* dimensions. The latter supposes that eelgrass pollen and flowers are adapted to submarine (*three*-dimensional) pollination. There is empirical evidence to support both hypotheses. This study examines the influence of tides on the presentation and dispersal of eelgrass pollen. To test the effect of two versus three-dimensional environments on dispersal, water samples containing pollen were compared as low tide approached and water volume decreased. To investigate evidence for adaptation to two versus three-dimensional environments on eelgrass flowering phenology, inflorescences were examined to determine their sexual stage during periods of extreme and weak tidal events. Results show that pollen concentration and the number of inflorescences at the water's surface decrease with water volume. Observations of flowering phenology show no difference in the activity of male and female flowers during differing tidal periods. These results suggest that timing of sexual activities is not coordinated with bi-monthly tidal events, and that a reproductive strategy relying on tidal fluctuations to concentrate pollen at the water's surface would result in the immobilization of some pollen. These results provide support for the three-dimensional pollination hypothesis, because pollen and receptive stigma interaction depends on water volume.

Introduction

Environmental forces that move pollen between individuals help to facilitate outcrossing in flowering plants. The pollen vector a plant uses depends upon the forces available in the habitat. Terrestrial plants rely on animals or wind to transport pollen, while aquatic plants may also rely on water (Barrett et al. 1993). The use of water as a vector for pollen (hydrophily) is mainly associated with submerged flowers, a trait which all seagrasses share (Den Hartog 1970; Sculthorpe 1967).

Hydrophily occurs by three principle modes: pollen is carried above the water's surface by accessory structures; pollen moves directly on the surface of the water; or pollen moves below the surface of the water (Cox 1988). Many plants are not limited to just one mode of hydrophily. Seagrasses typically employ one or both of those modes where pollen moves in direct contact with water. The transport of pollen on the two-dimensional surface of the water may be evolutionarily important, due to the increased efficiency of pollination occurring in two dimensions (Cox 1983). On the other hand, derived morphological traits suggest specific adaptation to subsurface pollination in some species, such as *Zostera marina* (Ackerman 1986; Ackerman 1997a; Ackerman 1997b). These apparently discordant hypotheses have resulted in controversy between their proponents (Ackerman 1995; Cox et al. 1992).

Cox (1983) put forward the random motion hypothesis. The random motion hypothesis is an intuitive, elegant and appealing application of search theory and the nature of random motion to hydrophilous pollen dispersal. Cox differentiated between two-dimensional and three-dimensional space for pollen dispersal. In two-dimensional space a randomly-moving object will contact every possible point, given enough time. An

object moving randomly in a three-dimensional space is not guaranteed to visit every point, no matter how much time passes; some points in a three-dimensional space will remain unvisited indefinitely. In effect, the time needed for a search vehicle to contact a particular point is shorter in a two-dimensional environment. Therefore, pollen dispersal in two dimensions will be more efficient than dispersal in three dimensions. Cox argues that the surface of the water provides an obvious plane of dispersal to hydrophilous plants. Individuals that disperse pollen when anthers and stigmas both occupy the water's surface then gain a selective advantage, compared to individuals dispersing pollen below the surface.

Ackerman (1986) investigated the effect of seagrass morphology on the flow of water as a pollen vector using experimental simulations. At the community level, Ackerman found that reproductive structures occurred at certain positions in the vertical water column where, for reasons related to fluid mechanics, effective pollination was more likely to occur. At the individual level, fluid flow was relatively uniform, compared to flow around the flowers of wind-pollinated species. A boundary layer effect occurs around inflorescences, possibly due to exerted stigmas. Ackerman suggested that these mechanical forces may act to concentrate male and female flowers together, and to allow stigmas to effectively filter pollen from the subsurface water column; movement of pollen would then be non-random in relation to inflorescences and exerted stigmas. Floral exertion reduced flow velocity but increased shear stress near inflorescences, creating a flow environment that was different from open water (Ackerman 1997a). Shear stress around inflorescences was found to cause individual filiform pollen grains to rotate and move along the inflorescence in a parallel fashion. Such movement, in relation

to the inflorescence, allows for multiple opportunities of pollen capture by exerted stigmas (Ackerman 1997b). Ackerman contrasts his conclusions with the ideas of Cox by describing them as "competing theoretical models" (Ackerman 1995). Ackerman goes on to identify five assumptions implicit in Cox's (1983) model, then argues that all five assumptions are violated. Ultimately, Ackerman claims that the theory of random motion is not applicable to hydrophilous pollination because pollen moves on or through water in a non-random fashion. For this reason, Ackerman concludes that surface pollination (i.e., during low tide) is relatively unimportant compared to subsurface pollination (Ackerman 1995).

The movement and capture of eelgrass pollen by receptive stigmas was observed under intertidal field conditions by Cox and co-workers (1992). They report frequent contact between pollen aggregations, or rafts, and exerted stigmas at the surface of the water. They do not observe any instances of subsurface contact between pollen rafts and stigmas, although they do not rule out the possibility of subsurface pollination in *Z. marina* (particularly in subtidal habitat). They criticize the work of Ackerman (1986), implying that his experimental simulation approach was not as satisfactory as their observations and the work of De Cock (1980). Specific criticisms include: no natural observations of pollen transport/pollination events; oversimplifying the movement of water currents at the surface of the water; and relying on poor analogues for real pollen. By comparison, Cox and co-workers used the observations of De Cock, who concluded that tidal events may play an important role in concentrating pollen and stigmas at the surface of the water. The results of Cox et al. support their surface pollination

hypothesis, but pollen observations made under more controlled conditions would add credibility to the assessment of surface versus submarine pollination hypotheses.

This study explores the relationship between tidal events and hydrophilous pollen dispersal in intertidal eelgrass of Humboldt Bay, CA, by capturing real pollen in its natural environment but quantifying it in the laboratory. Constant reduction of water volume in an eelgrass bed due to the approach of low tide may concentrate pollen and stigmas together, but sufficient loss of water will isolate pollen, so that it is unable to reach inflorescences other than the inflorescence which released it (Laushman 1993). This study tests the hypothesis that pollen concentration increases as water volume decreases by taking repeated measurements of waterborne pollen and surface inflorescences during the approach of low tide. The phenology of pollination is also investigated during periods of more and less extreme tide levels. Intertidal eelgrass habitat only infrequently experiences water levels low enough to concentrate many inflorescences at the surface. If eelgrass phenology is adapted to tidal cycle for the purpose of concentrating pollen at the surface, then more anthers should dehisce during periods of extreme tides than during periods of less-extreme tides. Finding greater flowering activity during periods of extreme tidal levels would suggest the adaptation of floral phenology to environmental cues.

Methods

Study system and site description

Zostera marina, marine eelgrass, is a monoecious, self-compatible seagrass that is common in the northern hemisphere (Den Hartog 1970; Ruckelshaus 1995). Flowers consist of either a bithecate stamen or a carpel that has a single style and two stigmas. All flowers lack a perianth. Individuals exhibit protogyny, at the inflorescence-level, by exerting stigmas of the female flowers between paired flaps ('spatha') that cover the inflorescence. After approximately 12 hours of pollen receptivity, during which time fertilization can occur, the stigmas of an inflorescence bend downward, under the spatha, and then abscise from the female flowers. The anthers of male flowers emerge in a similar fashion and dehisce, releasing filiform pollen anywhere from two to seven days after stigmas initially emerge. Fruits ripen and dehisce in approx. 30 days. Pollen threads are typically small, no more than a few mm in length. The functional lifespan of pollen threads has been estimated to be as long as 48 hours, but some pollen is non-functional at the time of dehiscence (De Cock 1980). Another study places pollen longevity more on the order of five hours (Cox et al. 1992).

Humboldt Bay is a significant eelgrass habitat in the eastern Pacific Ocean, and is the setting for the work described here. It is a sheltered embayment in northwestern California approximately 15 km long (north-south) and varying in width (west-east) from under one km to approximately five km. Channel dredging operations have sub-divided the mudflats in the interior of the bay into discrete parcels with evident edges at low tide. Mudflats in the interior of the bay are currently used for oyster cultivation, and historical

oyster cultivation methods (last employed in the 1970s) were highly disruptive to eelgrass habitats (J. Robinson, pers. comm.; S. Schlosser, pers. comm.). Brant and other migratory waterfowl that feed on *Z. marina* use the bay as a major staging area in the eastern Pacific flyway (Moore et al. 2003).

Field procedure

To examine the effect of daily low tide on pollen and stigma concentration, two consecutive days were chosen during a period of extreme tidal events in July, 2004. The estimated time of low-low tide at Fields Landing was determined for each day, using readily-available tide tables. Field procedures for the two days did not differ, although activities were conducted at different bed habitats (plots) on each day. Nine sampling points, positioned at regular (approx. 3m) intervals along the edge of the bed, were located prior to the start of sampling. All sampling was performed within one meter of the appropriate sampling point. Ninety minutes before the published time of low tide, a set of water samples and observations were made at each sampling point. A set of water samples consisted of two 25-ml scintillation vials filled with water collected at the surface, and two vials filled with water collected from the subsurface. To obtain a water sample, a capped vial was placed on its side at the appropriate level in the water column. The cap was then removed and, after five seconds, the open vial was capped and withdrawn from the water. If there was three centimeters or less of water at a sampling point, no subsurface water sample was collected at that time interval; no (surface or subsurface) samples were collected at a time interval if no water was present at all. Water samples were returned to the laboratory for quantification of eelgrass pollen-loads.

In addition to the water samples, observations made at each sampling point consisted of a measurement of depth (in cm), and a count of all reproductive shoots seen floating at (but not projecting above) the surface of the water. Sampling was repeated at 60 minutes prior to low tide, 30 minutes prior to low tide, and at the published time of low tide.

To examine the phenology of eelgrass, in relation to the larger-scale cycles of extreme and non-extreme tides, reproductive shoots were collected at two sites during extreme and non-extreme tidal periods. Approximately five reproductive shoots were gathered at each of nine sampling points (positioned as described above). All collected shoots were returned to the laboratory for inspection of their inflorescences.

Laboratory procedure

Water samples were poured from scintillation vials into clean plastic Petri dishes. Petri dishes were then placed so that any sediment would settle on the bottom of the dish. After settling occurred, the content of each Petri dish was examined for eelgrass pollen and pollen aggregations using a Leica MZ 8 binocular dissecting microscope. Pollen threads and pollen aggregations were tallied separately for each vial. Known eelgrass pollen was available for the purpose of comparison in ambiguous cases.

The inflorescences of reproductive shoots were examined to determine their reproductive status using forceps and a Bausch and Lomb 1x-2x binocular dissecting microscope. The spatha were pulled back to reveal a row of flowers or fruits. Inflorescences were sorted into one of four groups, depending on the presence of mature female or male flowers, or fruits. The ‘immature’ group had immature female and male flowers intact, or not fully formed. The ‘female’ group had erect, receptive female

flowers or recessed female flowers lacking stigmas, but immature male flowers with undehisced thecae. The 'male' group had exerted male flowers, either containing pollen or not. The 'fruiting' group had ovaries that had undergone some degree of darkening and enlargement.

Data analysis

Data collected during the approach of low tide was analyzed in a mixed model repeated measures design, using the statistical software package JMP IN v.5.1.2 (Sall et al. 2003). The mean numbers of surface and subsurface pollen, and the mean number of inflorescences, were assigned as response variables in separate analyses. For the analysis of pollen the model included the following effects: plot, location nested within plot, water depth nested within location and plot, sample position (i.e., surface or subsurface) nested within location and plot, and the interaction of depth and sample position. Plot, location, and depth were designated as random effects. The test of inflorescences was similar, but without the sample position effect. An analysis favors the random motion hypothesis if surface pollen and inflorescence concentrations increase with the arrival of low tide. An outcome would suggest no adaptation to surface pollination if there is no change or concentration decreases with low tide. Such a result would support the subsurface pollination hypothesis.

To test for a relationship between eelgrass phenology (i.e., reproductive condition of inflorescences) and tidal events, an ANOVA model was constructed with plot, inflorescence condition, tide status, and the interaction of inflorescence condition and tide status defined as main and interaction effects. Plot was treated as a random effect. A

finding of more dehiscent male and receptive female flowers during a period of extreme tides is evidence of adaptation to tidal cycle. No difference in floral condition between extreme and less-extreme tides, or fewer dehiscent/receptive flowers during extreme tides, suggests that floral phenology is not adapted to tidal cycle.

Results

The approach of low tide

Counts of water-borne pollen were generally low (all but one water sample contained either zero or one pollen search vehicles). Mean pollen concentration differs only by depth (Table 1), with pollen concentration decreasing with water depth regardless of surface versus subsurface sampling (Fig. 1). Only one sampled pollen search vehicle, out of 288 water samples, consisted of an aggregation of pollen threads; all others consisted of solitary pollen threads.

Low tide influences inflorescence number at the surface in much the same way that it influences pollen concentration. The mean number of surface inflorescences differs significantly for plot, location, and depth effects (Table 2). In general, the number of surface inflorescences declines as low tide approaches and water depth decreases (Fig. 2).

Eelgrass phenology and tidal cycles

Inflorescence sampling from two plots produced 21 immature, six female-flowering, one male-flowering, and 57 post-flowering inflorescences during the period of extreme tides, compared to 24 immature, nine female-flowering, zero male-flowering, and 54 post-flowering inflorescences during the period of non-extreme tides. The abundance and reproductive status of *Z. marina* inflorescences did not differ for extreme and non-extreme tidal periods ($p = 0.922$). Some sampled inflorescences bearing immature fruit were observed to retain pollen beneath the spatha. These inflorescences had clearly passed through the period in which female and male flowers were reproductively active; in some cases, the dehisced anthers were still intact and bearing pollen beneath the spatha. This result was not precisely quantified, but occurred in no more than eight inflorescences of 'fruiting' stage (out of 111 eligible samples).

Discussion

Conflicting hypotheses propose that eelgrass is adapted to two-dimensional pollination in which tidal phenomena play a facilitating role, and to three-dimensional pollination involving the non-random movement of pollen due to morphological adaptations. The concentration of waterborne pollen decreases as water volume (i.e., water depth) decreases leading to low tide. This occurs because water completely disappears from some sampling points. The same is true of the decreasing water volume's effect on the number of surface inflorescences; the decrease does not effectively concentrate them, so much as render them unreachable by pollen. There is

also a general scarcity of pollen during the height of the reproductive season and in particular the near-absence of pollen aggregations. While there is a tidal effect on the concentration of pollen and inflorescences, the decrease observed does not support adaptation to surface pollination or pollination during low tide in *Z. marina*.

Comparisons of floral phenology of reproductive activity showed no differences between periods with low tides of relatively extreme and of modest magnitudes. This result suggests that bi-monthly tidal cycles do not influence the timing of flowering events in *Z. marina*. There is no evidence of adaptation to bi-monthly tidal cycles, and no reproductive advantage via the timing of flowering events to coincide with periods of extreme tides can be inferred.

Search theory and Cox's random motion hypothesis (1983) might be one contributing force in the dispersal of *Z. marina* pollen, if tide were not important. However, *Z. marina* occupying the intertidal habitat may not be ideally suited to having its pollen most abundant in the environment during extreme low tides. This is because of the likelihood that water is unavailable at such times. Even when pools of standing water remain at low tide, released pollen will likely disperse to another inflorescence belonging to the same plant, or to those of near-neighbors. This scenario suggests how a plausible condition in the physical environment, such as low tide, may lead to geitonogamy and the establishment of fine-scale population structure, due to the diminished arenas of pollen dispersal. During such periods there would be no subsurface pollination, but conditions would also preclude the possibility of pollination on the surface in many cases. Since extreme low tides occur infrequently over the course of a month, any adaptive advantage they provide would have to be convincingly strong. The observed results do not support

the hypothesis that efficient pollination is likely to occur in the intertidal zone during low tide.

Ackerman's subsurface pollination hypothesis (1986; 1997a; 1997b) emphasizes how the pollen and inflorescence morphology of *Z. marina* appears precisely adapted to moving through three-dimensional space in a non-random fashion, and to successfully facilitating encounters between pollen and stigmas. Results presented here favor the possibility of subsurface pollination when water volume is plentiful because in some situations (e.g., the intertidal zone during low tide) there is a risk of interruption to the pollen vector. The lack of apparent phenological timing in response to bi-monthly periods of tidal extremes suggests that widespread propagule release during a period when tides reach their lowest point is not an adaptive trait. The possibility of subsurface pollination in ample water volume exists in and out of such periods, so no preference in the timing of pollen release and stigma receptivity means that there is no adaptation to tide-mediated pollination in Humboldt Bay marine eelgrass.

The overall scarcity of pollen and pollen aggregations in surface and subsurface samples may limit the generality of the results presented here. The scarcity of pollen observed here is different from Cox et al. (1992), who observed abundant pollen aggregation and movement in the field. The methods used here were intended to improve on the method of Cox et al. Their method of field observation seems unreliable because of the small size of pollen threads and the constant movement of water. Poor pollen representation in our method may have occurred because the water samples were too small. Another possible reason for pollen scarcity might be flowering phenology. Pollen collection occurred in the middle of summer, when reproduction should have been

actively occurring. A comprehensive study of flowering phenology throughout the growing season, incorporating the collection of water samples for analysis of pollen loads, would help resolve this question. The possibility of severe pollen reduction in the water column due to filtering by receptive stigmas seems unlikely, considering that an inflorescence contains almost twice as many male flowers as female flowers, and each male flower contains hundreds of pollen (J. Neely, unpublished data).

The results of this study do not unequivocally distinguish between the random motion and the submarine pollination hypotheses. However, limitations to propagule dispersal at low tide imposed by the frequent absence of water at intertidal eelgrass beds and the lack of coordination between flower phenology and periods of extreme tides are more consistent with Ackerman's hypothesis. For these reasons, we conclude that the drawbacks to pollinating at low tide may constrain adaptation when pollen is either stranded or when geitonogamy occurs, while the infrequent occurrence of extreme low tides in intertidal habitats do not influence the timing of flowering. The prevailing condition of ample water availability would not constrain the morphological adaptation of pollen threads and inflorescences.

Acknowledgements

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Tables

Table 2.1. ANOVA of *Z. marina* mean pollen concentration collected at Humboldt Bay, CA. Plot refers to two different plots sampled on consecutive days. Location[Plot] indicates that sampling locations are nested within plots. Depth[Plot, Location] indicates that the recorded depth is nested within plot and sampling location. Sample position[Plot, Location] indicates that the position of sampling in the water column is nested within plot and location. Sample position*Depth indicates the interaction of the listed main effects. Pollen concentration varies by water depth ($p < 0.05$).

Source	Sum of squares	Mean squares	Degrees of freedom	F Ratio	Prob > F
Plot	0.068	0.068	1	0.541	0.463
Location[Plot]	2.438	0.152	16	1.282	0.211
Depth[Plot,Location]	3.796	0.211	18	1.779	0.029
Sample position[Plot,Location]	1.331	0.074	18	0.624	0.879
Sample position * Depth[Plot,Location]	1.602	0.089	18	0.751	0.756

Table 2.2. ANOVA of *Z. marina* mean surface inflorescences sampled at Humboldt Bay, CA. The descriptions of the model effects are the same as in Table 1. Surface inflorescences vary by plot, location, and water depth ($p < 0.05$).

Source	Sum of squares	Mean squares	Degrees of freedom	F Ratio	Prob > F
Plot	56.018	56.018	1	8.684	0.006
Location[Plot]	310.608	19.413	16	7.110	<.0001
Depth[Plot,Location]	201.748	11.208	18	4.156	<.0001

Figures

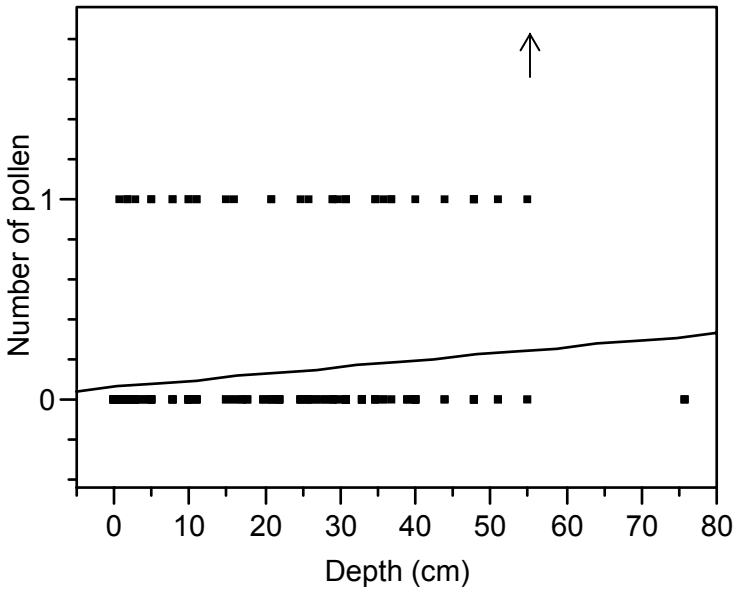

Figure 2.1. Relationship between pollen concentration and water depth in a *Z. marina* eelgrass bed at Humboldt Bay, CA. Water depth (in cm) is on the X-axis and the mean number of pollen threads per water sample is on the Y-axis. The line represents the linear regression of pollen count on water depth. The concentration of waterborne pollen is reduced when water depth is low ($p = 0.029$; see Table 1). The arrow indicates a data point (not shown) at $y = 2$.

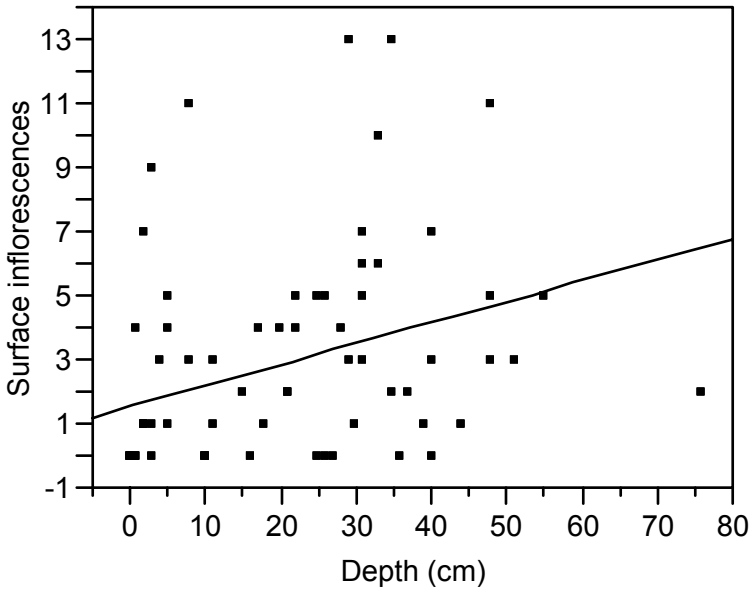

Figure 2.2. Relationship between inflorescences at the surface and water depth in a *Z. marina* eelgrass bed. Water depth (in cm) is on the X-axis and the mean number of surface inflorescences is on the Y-axis. The line represents the linear regression of surface inflorescences on water depth. Inflorescences in contact with the water's surface are less plentiful when water depth is low ($p < 0.0001$; see Table 2).

CHAPTER THREE: FINE-SCALE VARIATION OF REPRODUCTIVE STRATEGY

Abstract

Habitats that exhibit stark dissimilarity over a localized area may produce sufficiently strong forces of selection to cause adaptation at fine spatial scales. Adjacent intertidal and subtidal habitats in bays along the NW coast of North America differ in the amount of time each habitat is exposed to terrestrial and aquatic conditions. This study considers reproductive strategies of *Zostera marina* in adjacent intertidal and subtidal habitats by sampling from nine separate populations located in the eastern Pacific Ocean. Shoot density and mass were measured at intertidal and subtidal sites in nine bays, and microsatellites were used to produce multi-locus genotypes for sampled ramets. Vegetative shoots were found to be smaller but more densely arranged in the intertidal habitat. No difference between habitats was found for reproductive shoots or for clonal diversity. Single- and multi-locus genetic analysis showed little population structure between habitats. Results may suggest that *Z. marina* reproductive strategy does not differ between habitats, within the portion of the species range sampled. Recruitment from seed may occur infrequently, but it appears to be more heavily relied upon than recruitment through clonal growth. Since plant vegetative features did differ between habitats, there may possibly be other unobserved features of the breeding system that are selected upon by habitat.

Introduction

Growth and reproduction are two important life history components for organisms to allocate effort and resources (Harper 1977). Differential allocations to clonal growth and sexual reproduction may represent reproductive strategies that vary by environmental conditions present (De Jong et al. 1999; Eckert et al. 1999). Examples of local differentiation are particularly interesting, because environmental forces that shape phenotypic expression must be strong and directly observable in order to overcome the homogenizing forces of gene flow and clonal spread (Kingsolver et al. 2001; Slatkin 1987). A classic example of local differentiation in plants involves discrete edaphic boundaries formed by mine tailings containing elevated levels of heavy metals (Antonovics 1967). Copper-tolerant plants grow on mine-tailings but are not found on adjacent uncontaminated soil. Copper-tolerant plants also self-fertilize more frequently than plants on uncontaminated soil, even in the face of inbreeding depression. This mating system difference establishes a barrier to gene flow, and may facilitate local adaptation and, eventually, speciation. The coastal intertidal zone is a discrete natural ecotone, analogous to the ecotone generated by mine tailings. The coastal intertidal thus provides a natural system for studying phenotypic expression of reproductive strategies, and for documenting genetic differentiation of closely-adjacent habitats.

Most clonal plants adopt reproductive strategies that combine sexual and asexual modes of reproduction (Handel 1985; Silander 1985). Individual reproductive strategies may result from many different evolutionary forces. One common hypothesis, made in particular reference to the marine aquatic environment, is that sexual reproduction is

selectively advantageous in the highly disturbed intertidal compared to the more stable subtidal environment (Hughes and Stachowicz 2004; Les 1988; Reusch et al. 2005). One putative advantage to increased sexuality in disturbed environments is the production of a diverse array of progeny genotypes resulting in at least some progeny capable of persisting in conditions that are different from those experienced by the parents (Barton and Charlesworth 1998). A second hypothesis is that clonality offers a form of assured persistence under conditions where sexual reproduction is unreliable (e.g., at the margin of species' geographic ranges) (Eckert 2001). Similar reasoning applies to subtidal individuals of *Z. marina*, where the large volume of water may make sexual reproduction very inefficient (Cox 1983).

There are important differences in the reproductive ecology of intertidal and subtidal *Z. marina*. For instance, flowering frequency can differ between habitats and at different latitudes across the species range, suggesting dissimilar reliance on sexual reproduction (Phillips et al. 1983a). Dichogamy is more pronounced in intertidal habitats; this corresponded with a higher outcrossing rate (Ruckelshaus 1995). Geographically marginal eelgrass populations exhibit greater reliance on clonal reproduction in some (Billingham et al. 2003; Reusch et al. 1999a) but not all studies (Phillips et al. 1983a). Corresponding to differences in reproductive ecology, differentiation at allozyme loci exists between local intertidal and subtidal habitats (Ruckelshaus 1998), and greater dichogamy corresponds to a higher outcrossing rate (Ruckelshaus 1995). A combination of ecological data measuring the density and size of reproductive and vegetative ramets and molecular data measuring the single- and multi-

locus genetic differentiation provides an opportunity to integrate comparison of the reproductive strategies and genetic differentiation of *Z. marina* in different habitats.

The objective of this study is to quantify differences in reproductive strategy among intertidal and subtidal habitats of the seagrass *Zostera marina*. To investigate whether tidal habitat influences aspects of reproductive strategy, we collect ecological data on ramet size and density at intertidal and subtidal locations in nine large Z. marina populations along the NW coast of the United States. If strategy varies by tidal exposure at a fine scale, then we expect phenotypic expression will differ between habitats. To investigate the scale of genetic differentiation, we measure single- and multi-locus genetic differentiation between habitats and at larger geographic scales. Results showing more prolific growth of reproductive shoots and higher clonal diversity in the intertidal habitat would support the hypothesis that disturbance leads to increased sexual reproduction, while results showing less evidence of sexual reproduction and lower clonal diversity in the intertidal would suggest a clonal growth strategy in response to unfavorable conditions.

Methods

Study System

Zostera marina L. (marine eelgrass) is a temperate-zone seagrass that grows in intertidal and subtidal habitats. The reproduction of *Z. marina* is characterized by clonality (via rhizomatous growth), monoecy, protogyny (De Cock 1980), and limited dispersal distances by water of sexual propagules (Orth et al. 1994; Ruckelshaus 1996). Growth by rhizomes allows perennial life spans in most populations that are not

subjected to freezing or other severe weather (Den Hartog 1970). Local clonal structure influences the mating system by encouraging geitonogamy (Reusch 2001a; Reusch et al. 2000). Local adaptation has been demonstrated between Baltic Sea populations *Z. marina* separated by a distance of 50 km (Hammerli and Reusch 2002).

Samples of *Z. marina* were taken from nine populations located in the eastern Pacific Ocean across three US states: California, Oregon, and Washington. California populations consisted of (from south to north) Mission Bay (Mis), Tomales Bay (Tom), Bodega Bay (Bod), and Humboldt Bay (Hum). Oregon populations consisted of Coos Bay (Coo) and Tillamook Bay (Til). Washington populations consisted of Willapa Bay (Wil), Case Inlet (Cas), and Padilla Bay (Pad). Figure 1 shows the locations of all nine sample sites. The sampling area represents the southern and central portions of the species range exhibited in the eastern Pacific Ocean (Den Hartog 1970; Phillips et al. 1983b). At low tide, intertidal and adjacent subtidal habitat occupied by *Z. marina* was present at each site, except Willapa Bay, where subtidal habitat was scarce. Exotic *Z. japonica* was observed in or near *Z. marina* habitat at the following sites: Case Inlet, Coos Bay, Padilla Bay, and Willapa Bay.

Data Collection

Field Procedure

Populations were visited one time during morning low tide from June to August, 2005. This sampling schedule was used to maximize the likelihood of sampling during the peak reproductive season (Phillips et al. 1983a; Phillips et al. 1983b); populations were visited only once because of the practical limitations imposed by sampling

geographically disparate populations at extreme low tide. Four accessible sampling 'locations' were established at each population (except at Willapa Bay and Case Inlet, where limited availability of continuous habitat meant that only two and three locations, respectively, were established). Locations were marked at 40-m intervals along the low-tide water line. The initial location was chosen based on the presence of *Z. marina* individuals and then the following three locations were placed based on measurements. Each location consisted of two 'habitat flags' placed on either side of the low-tide water line. One flag was in intertidal habitat and the other in subtidal habitat. The flags at a location were placed approximately 10 m apart, to represent the size of the genetic neighborhood of *Z. marina* (Hammerli and Reusch 2003a). Genetic neighborhood likely varies due to local conditions. In fact, Hammerli and Reusch's estimate is conservative; a previous estimate of genetic neighborhood in the species was approx. four times larger (Ruckelshaus 1996).

Two 0.1 m^2 quadrats were anchored to the substrate on either side of each habitat flag. Small quadrats were used to keep the amount of biomass gathered to a manageable level. All *Z. marina* ramets located in the quadrats were severed at the level of the substrate and stored in silica gel desiccant for transportation to the laboratory. The numbers of vegetative and reproductive ramets collected in each quadrat were used to measure density.

Laboratory Procedure

Once in the laboratory, desiccated ramets were rinsed to remove silica gel crystals. Epiphytic algae present on the samples were removed by hand. Vegetative and reproductive shoots were sorted for weighing. The chance of incorrectly sorting a shoot is unlikely since reproductive shoots exhibit a gross morphology that is different in leaf-blade structure from vegetative shoots. This feature is useful when sorting immature reproductive shoots without well-developed inflorescences. The ramets were then dried in an oven at 60° C for 48 h. Dried tissue was weighed on a digital balance and discarded.

Ecological data consisted of measurements of density and dry mass of vegetative and reproductive ramets collected in each 0.1 m^2 quadrat. The number of each type of ramet (vegetative or reproductive) collected in a quadrat determined the densities for a quadrat. A mean value for each habitat flag (i.e., averaging over two quadrats) was calculated for each of the measurements: vegetative density, vegetative mass, reproductive density, and reproductive mass. Relative reproductive density using mean values of density was also calculated.

Prior to drying, approximately 0.4 g of tissue was removed from some ramets for molecular analysis. Four published *Z. marina* loci (CT-12, CT-20, GA-2, and GA-3) (Reusch 2000a; Reusch et al. 1999b) were used for microsatellite analysis. For each habitat flag (where two quadrats were used) up to six ramets were analyzed for genotype, for a study-wide total of 325 analyses. Ideally, three ramets from each quadrat were used to reach the goal of six ramets, but an unequal number of ramets from each quadrat at a

habitat flag were sometimes used. Habitat flags with a pooled number of ramets less than six occasionally occurred in the data set. Preparation of DNA samples for microsatellite analysis and scoring of genotypes followed the procedure described in Chapter 1.

Data Analysis

Density and mass of vegetative and reproductive ramets were analyzed with the JMP IN software package (Sall et al. 2003). Effects included in the mixed model were population, location nested within population, and habitat nested within population. All of the effects, except location, are considered as fixed effects in the analysis. Sampling was performed at some populations during the new moon phase and at others during the full moon phase. The severity of low tides tends to be greater when the moon is full, so the habitat treatment cannot be considered identical. For response variables with significant results, the effect of moon phase at the time of sampling was tested with one-way ANOVA where two levels, 'new' and 'full', for the variable 'moon phase' were used.

Population genetic data consisted of four-locus genotypes for every ramet sampled. In some cases the genotypes at one or more loci were equivocal and could not be scored. Clonal diversity at each habitat flag was calculated using Simpson's diversity index (Hangelbroek et al. 2002) and the diversity index used by Ellstrand and Roose (1987). These indices differ in the extent they account for relative frequency of genotypes. See Chapter 1 for an explanation of each index. Mixed model ANOVA was used to compare clonal diversity, with main effects the same as those described above. The GDA software package (Lewis and Zaykin 2001) was used to estimate and calculate

bootstrap values across loci for co-ancestry coefficients in a four-level genetic analysis of the entire experimental design (Weir 1996). Hierarchical *F*-statistics were calculated from these estimates of co-ancestry for the different levels of the experimental design. The calculation of hierarchical *F*-statistics is the division of variance at a level of hierarchy by the additive variance at a more inclusive level of hierarchy. Bootstrapping of hierarchical *F*-statistics used 40 replicates. A series of two-level genetic analyses involving the intertidal or subtidal samples of single populations were also conducted to allow the comparison of inbreeding between habitats and among populations.

Results

Vegetative and Reproductive Measurements

Average vegetative and reproductive measurements are summarized in Table 1. Reproductive shoots were scarce at all populations, compared to vegetative shoots. The scarcity of reproductive shoots among all populations and habitats was unexpected since sampling occurred at the height of the reproductive season in mid-summer. Mixed model analysis of density and mass of vegetative and reproductive shoots produced varied results. Significant differences ($p < 0.05$) were found for vegetative density and vegetative mass (Table 2) at the levels of population and habitat (higher density and lower mass at intertidal locations). Reproductive density and mass did not differ by habitat ($p = 0.690$ and 0.596, respectively) or by any of the other effects included in the model. Relative reproductive density also did not differ by habitat ($p = 0.330$) or any other effect. The small number of reproductive shoots observed means that statistical power for these tests is limited. Figure 2 shows the distribution of mean values for those

traits exhibiting significantly different measurements, as well as mean reproductive mass and relative reproductive density. In each case only some of the habitats differ (i.e., non-overlapping error bars in Fig. 2); the identities of populations with differing habitat effects are not consistent between density and mass. Heterogeneity demonstrated by extreme values (e.g., Bodega Bay intertidal for mean vegetative density and Tilamook Bay subtidal for mean vegetative mass) likely contribute disproportionately to the significantly-different statistical outcome for habitat (Table 2).

Populations were sampled during low tides associated with the new or full moon, but not both. Analyses of variance for the effect of moon phase on vegetative response variables showed that responses did not vary by moon phase (mean vegetative density, $p = 0.846$; mean vegetative mass, $p = 0.517$).

Population Genetics

Microsatellite analysis revealed five alleles at the CT-12 locus, six alleles at the GA-3 locus, and seven alleles at each of the CT-20 and GA-2 loci, over all populations sampled. Each allele did not appear in every population, suggesting some structure among populations. Table 3 summarizes the number of alleles and average heterozygosity for each locus and population. Genotype data for some individuals, constituting approximately 10 percent of the total number of samples analyzed, was not included in some of the following analyses due to equivocal identity resulting from one or more unresolved loci.

Table 4a shows estimates of co-ancestry coefficients and bootstrap analyses calculated for the entire system under study. Table 4b shows *F*-statistics for the

hierarchical components of the experimental design, calculated from the co-ancestry coefficients. A value of zero for any hierarchical *F*-statistic indicates close correlation between heterozygosity values at the levels of the hierarchy being compared. As an estimate approaches one, the proportion by which heterozygosity is reduced is indicated. Another way of interpreting estimates is the degree of allelic relatedness present at different levels of the hierarchy. Four-level analysis of genetic variance produced estimates of differentiation between individuals within habitats (inbreeding within populations, the parameter *f*), differentiation between individuals over all populations (the parameter *F*), differentiation between habitats within genetic neighborhoods, differentiation between genetic neighborhoods within populations, and differentiation between populations (the parameter θ_P) (Weir 1996). Bootstrap analysis of co-ancestry coefficients and hierarchical *F*-statistics across loci provided 95% confidence intervals for these parameters; confidence intervals that do not overlap with zero provide statistical support ($p < 0.05$), demonstrating that the estimate is different from zero. Overall values for all hierarchical *F*-statistics are statistically different from zero, except for that at the level of between genetic neighborhoods within populations. The actual estimates of *F*-statistics appear heterogeneous for individual loci; the estimates of all parameters for the CT-20 locus appear lower than those for other loci. This result may have occurred due to limited unequivocal genotypes for the locus or scoring inconsistencies such as a feature of the background noise being misidentified as an allele. However, all loci were scored in a consistent and proven manner.

The comparison of inbreeding (*f*) between habitats and populations is shown in Fig. 3. Each habitat in every population studied has a 95% confidence interval of *f*-

values provided. Confidence intervals that overlap with each other indicate that inbreeding does not differ between the habitats or populations being compared. Estimates of *f* fall between 0.4 and 0.8 for all habitats and populations except Humboldt Bay. While Humboldt Bay estimates appear lower, they also exhibit larger confidence intervals. In only a few instances do confidence intervals not overlap with that of the Humboldt Bay intertidal estimate (e.g. Coos Bay intertidal and subtidal estimates).

Analysis of multi-locus microsatellite genotypes revealed that samples contained predominantly different, unique ramets. This result causes clonal diversity to be equal to or near one for most locations, according to both indices used. Fig. 4 shows mean clonal diversity of intertidal and subtidal habitats for some populations. Data from Mission, Tilamook, and Willapa bays was omitted because these populations had fewer than four mean ramets per quadrat pair contributing to calculations of clonal diversity. For this study, fewer than four ramets gathered in a 1 m radius were considered insufficient for a reasonable estimate of clonal diversity. Out of those populations included, only two (Humboldt and Bodega Bays) exhibited more than a single sampling location (within a pair) in which clonal diversity was different than one. Such an observation suggests that few ramets are produced through clonal growth in either habitat. An alternative conclusion where diverse clonal propagules, such as rhizome fragments, are dispersed during a physical disturbance event is very unlikely due to the low frequency of successful establishment by such propagules in *Z. marina* (Ewanchuk and Williams 1996). Multi-locus genotypes compared between intertidal and subtidal habitats at the same populations included above (n = 39 sample locations) showed no difference in mixed-model ANOVAs of clonal diversity for each index (Simpson's: $p = 0.113$;

Ellstrand and Roose: $p = 0.202$). Distribution of means is assumed to not follow a normal distribution, based on the number of populations where clonal diversity = 1. This may violate an assumption of normal distribution in ANOVA, but additional analyses of just the Humboldt and Bodega Bay populations (see below) indicate that the violation does not influence the outcome significantly.

Further analysis between habitats at the Humboldt and Bodega populations (n = 16 sample locations), and within each of these populations by itself (n = eight sample locations each), also show no differences in clonal diversity between intertidal and subtidal habitats (Humboldt + Bodega: $p = 0.298$; Humboldt: $p = 0.593$; Bodega: $p = 0.355$).

Discussion

A fine-scale investigation of size and spatial density of reproductive and vegetative ramets, as well as genotypic identity, of *Z. marina* in subtidal and intertidal habitats was conducted. Growth and demography of *Z. marina* varies among discrete habitats created by tidal events; intertidal locations exhibit smaller but more densely-arranged individuals than subtidal locations (Table 1 and 2; Figure 2). This effect of habitat conditions at a fine scale does not extend to reproductive strategy and allocation of resources to reproduction: The size and density of reproductive shoots does not differ among intertidal and subtidal habitats. Also, clonal diversity does not differ among habitats. These results suggest that tidal influence affects growth, but modes of sexual and asexual reproduction do not differ at the fine scale tested here.

Bootstrap analyses of *F*-statistics show significant differences from zero at various scales of population organization (Table 4). Hierarchical *F*-statistics calculated from co-ancestry coefficients show a pattern where fine-scale variation (between individuals within habitats) is far greater than the variation found at other levels of the hierarchy (Table 4b). Such a result agrees with findings from an intra-population analysis of molecular variance in which little variation was detected at all but the finest scale surveyed (Chapter 1). Estimated variation between habitats is of particular interest here. Genetic variation between the intertidal and subtidal habitats is low, within genetic neighborhoods, but statistically different from zero. Since there is little evidence of clonal growth in either habitat, this slight difference may result from the dispersal of pollen and seed across habitat boundaries, creating similar patterns of relatedness in intertidal and subtidal habitats. Other studies of pollen dispersal in *Z. marina* have suggested that features of the intertidal habitat may help or hinder the effective dispersal of pollen (Cox et al. 1992; De Cock 1980). Forces contributing to patterns of propagule dispersal between habitats may also be responsible for genetic structure at finer scales. For example, in areas of high clonal diversity, self-fertilization (shown by variation between individuals within habitats) would occur predominantly within a single reproductive shoot. This is different from the widespread idea that geitonogamy in clonal plants usually occurs between sister ramets (Eckert 2000; Handel 1985; Reusch 2001). Comparisons between location pairs represent separate genetic neighborhoods, as estimated by Hammerli and Reusch (2003a). Our results indicate little structure between location pairs, suggesting that in the populations under consideration here the genetic neighborhood size is larger than the previous estimate. Moderate variation between

populations likely occurs due to the limited dispersal distances of reproductive propagules (Laushman 1993; Olsen et al. 2004; Orth et al. 1994; Ruckelshaus 1996).

The study-wide estimate for the inbreeding coefficient (*f* and variation between individuals within habitats) was high. A look at inbreeding separately for each population (Fig. 3) shows little significant difference but an interesting pattern. Those populations with the largest variance (Humboldt and Bodega Bays) for *f* also were those that showed evidence of being less than completely clonally-diverse. While differences in neither data set are supported by statistical significance, the correlation of trends noted here contradicts the logic that inbreeding should be more likely when clonality is prevalent. Also, Humboldt Bay was the only population to exhibit any instances of heterozygote excess. This result agrees with those of Hammerli and Reusch (2003b), that found a significant positive correlation between observed heterozygosity and clonality. They concluded that outbred clones become widespread, especially under regimes of low disturbance, due to competitive exclusion of relatively inbred neighbors. Although questionable, considering the dissected physical nature of Humboldt Bay (Chapter 1), our results may indicate that Humboldt Bay is less disturbed than many of the other populations studied.

The results of our study disagree with previous studies of reproductive strategy in *Z. marina* in some respects. An intra-population study conducted by Harrison and Durance (1992) included intertidal and subtidal sites. They found that plants at intertidal sites (regarded by the authors as less-favorable habitat) were smaller and less dense than plants at subtidal sites. While the response in plant size matches that presented here, the response in density is reversed. Since their study considered only one population, the

difference in the results could be explained by some attribute specific to their site, such as a particular scarcity of resources. Harrison and Durance also found that subtidal sites exhibited the lowest clonal diversity, and interpret this as evidence of increased reliance on sexual recombination at the intertidal sites. In contrast, we found generally high clonal diversities, regardless of habitat. Another study, conducted by Phillips et al. (1983a), related habitat variation to reproductive traits. They found evidence of greater allocation to reproduction in intertidal habitats, where *Z. marina* was more likely to exhibit an annual life history. In stable subtidal habitats, allocation to reproduction was low. Phillips et al. inferred that subpopulations maintain themselves through clonal growth. Their results differ from those of the current study, where allocation to reproduction and clonal growth does not appear to differ between habitats.

Hammerli and Reusch (2002) provided a successful test of local adaptation between two distant *Z. marina* populations. Their study involved careful reciprocal transplantation of consistent genotypes over a distance of 50 km and they demonstrated a preference of the home site in observed genotypes. Despite the lack of a hard test of fitness, the authors rationalize that their experiment represents an effective test of local adaptation. This represents a different approach to the analysis of habitat variation from the current study and each one offers different features. While they make no effort to distinguish sexual function in their experiment, Hammerli and Reusch discuss the possible influence of ramet density on allocation of growth resources; they infer that intraspecific interactions within the genetic neighborhood contribute to environmental patchiness more than abiotic forces. The current study was focused at the scale of the genetic neighborhood, where it overlapped the boundary between habitats. Our result of

larger shoots in the less-dense subtidal agrees with their finding that plants in areas of lower intraspecific competition allocate to shoots instead of rhizomes.

Neither of the underlying hypotheses used to explain patterns of reproductive strategy in seagrasses neatly fit the results presented here. If sexual reproduction is a response to disturbance or an unsuitable habitat (Hughes and Stachowicz 2004; Les 1988; Reusch et al. 2005), then simultaneous results of high clonal diversity and low sex expression across habitats appear contradictory. The presence of very few clonal ramets in local areas indicates the occurrence of sexual reproduction. On the other hand, little evidence of sex expression implies that sexual reproduction may be infrequent. Such observations weaken the validity of this hypothesis because they suggest that both habitats can be viewed as stable and suitable, where individuals can be recruited from seed yet engage in sex infrequently. Also, such individuals are likely long-lived, which would also discredit the idea that they exist in a stressful habitat to which they are poorly-suited. On the other hand, if conditions render sex unreliable and require prevalent clonality for population maintenance (Eckert 2001), the high overall levels of clonal diversity here suggest sexual reproduction is frequently relied upon.

Some limitations of the current study include the scarcity of reproductive shoots and the consideration of few morphological characters. The decision to sample spatial density and dry mass were made with the between-habitat scale of interest in mind, and also with particular interest in allocation to either clonal growth or sexual reproduction (as opposed to allocation between male and female aspects of sex). A previous pilot study conducted only at the Humboldt Bay population showed no suggestion of inflorescence number or seed mass as differing significantly between habitats (J. Neely,

unpublished data). The scarcity of reproductive shoots may have been alleviated in most populations if reproductive shoots were sampled in a different manner than the vegetative shoots. In every population except the Case Inlet population, reproductive shoots were observed even if none were sampled from the quadrats. The small size of the quadrats was necessary to limit the accumulation of matter over the course of the experiment, but they seem to have been inadequate for effectively sampling reproductive shoots. The non-significant trend where reproductive traits are favored in the intertidal habitats of southern populations (Mission, Bodega, and Tomales Bays) and favored in the subtidal habitats of more central populations (Coos and Tilamook Bays) may suggest agreement with the observations of Phillips et al. (1983a), in which Mexican populations at the southern extreme of the species range exhibited more prolific sexual reproduction than other regions. Related to the overall scarcity of reproductive shoots is the scarcity of shoots of either condition in certain populations, such as Willapa Bay. While the small quadrats allowed the sampling of adequate biomass in many populations, they were inadequate in some situations where plants were sparse. The problem was exacerbated at times by the need to exclude equivocal genotypes from the data set. Beyond these immediate concerns, this study does not represent a robust test of adaptation. Although initial inspiration for the questions stated here was drawn from a study of reproductive isolation in plants growing on mine tailings (Antonovics 1967), the current study represents observations of genetic and morphological variation that can be attributed to reproductive allocation strategies. However, an effort to separate phenotypic plasticity from adaptive responses in observed differences would only be limited to the

significantly-different vegetative characters, which would not reflect strongly on the evolution of reproductive strategies.

The conclusions presented here suggest that while sexual reproduction must play a significant role in *Z. marina* populations, regardless of habitat, no evidence of morphological differences in reproductive strategy between habitats exist. Genetic structure between habitats was found to be present at low magnitude, suggesting that reproductive barriers to gene flow may be real but weak within populations. *Z. marina* of the eastern Pacific Ocean appears to be a relatively-sexual clonal plant species that exhibits a great amount of inbreeding, yet is able to effectively disperse sexual propagules beyond the accepted scale of the genetic neighborhood. The clonal life history of *Z. marina* deserves more attention, based on the results shown here. With so little clonal reproduction as many populations show, the perennial life span of the species may be questioned at those locations.

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Tables

Table 3.1. Summary of average measurements, by population and habitat, for mean vegetative density (MVD), mean vegetative mass (MVM), mean reproductive density (MRD), and mean reproductive mass (MRM). For each measurement, the first number given indicates the intertidal ('Inter") average and the second indicates the subtidal ('Sub') average. Units for measurements of density are in number of shoots per 0.1 m^2 quadrat and measurements of mass are in grams.

	MVD		MVM		MRD		MRM	
	Inter	Sub	Inter	Sub	Inter	Sub	Inter	Sub
Bodega Bay	17.375	4.250	0.904	1.931	0.125	0.125	0.036	0.104
Case Inlet	5.667	3.000	0.199	0.144	0	0	0	0
Coos Bay	2.500	2.500	1.081	2.007	0.375	0	0.117	0
Humboldt B.	2.125	2.375	3.341	2.197	0	0	0	0
Mission Bay	8.125	2.875	1.175	1.046	0.250	0.125	0.022	0.052
Padilla Bay	4.625	3.000	1.694	1.938	0	0	0	0
Tilamook B.	1.625	2.000	1.399	6.476	0.125	0	0.243	0
Tomales Bay	3.125	2.000	0.266	0.830	0.125	0.125	0.008	0.103
Willapa Bay	1.000	3.000	1.052	2.809	0	0	0	0

Table 3.2. Mixed-model ANOVA of *Z. marina* (a) Mean Vegetative Density and (b) Mean Vegetative Mass collected at nine eastern Pacific populations. 'Population' refers to the populations sampled from. 'Pair[Population]' indicates the pairs of intertidal and subtidal sampling locations are nested within populations. 'Habitat[Population]' refers to the intertidal and subtidal habitats sampled from, also nested within populations. Mean vegetative density varies by habitat and population ($p < 0.05$).

a. Mean Vegetative Density

Source	DF	Sum of Squares	F Ratio	Prob > F
Population	8	503.299	17.772	<.001
Pair[Population]	24	115.458	1.359	0.229
Habitat[Population]	9	422.542	13.263	<.001

b. Mean Vegetative Mass

Source	DF	Sum of Squares	F Ratio	Prob > F
Population	8	77.721	3.900	0.007
Pair[Population]	24	64.957	1.474	0.174
Habitat[Population]	9	61.882	3.744	0.005

Table 3.3. Number of alleles and mean observed heterozygosity, by locus and population.

	Alleles per locus				Heterozygosity			
	CT-12	CT-20	GA-2	GA-3	CT-12	CT-20	GA-2	GA-3
Bodega Bay	2	4	3	4	0.200	0.286	0.086	0.314
Case Inlet	4	6	6	5	0.258	0.355	0.226	0.290
Coos Bay	5	5	7	5	0.091	0.152	0.152	0.212
Humboldt Bay	4	2	4	3	0.069	0.483	0.172	0.069
Mission Bay	3	5	3	3	0.185	0.482	0.185	0.111
Padilla Bay	4	6	5	5	0.167	0.333	0.200	0.367
Tilamook Bay	4	5	3	4	0.364	0.273	0.182	0.364
Tomales Bay	5	6	5	6	0.300	0.350	0.250	0.250
Willapa Bay	5	6	5	4	0.333	0.333	0.333	0.444

Table 3.4. *F*-statistics for a four-level hierarchical population analysis. (a) Coanecestry coefficients; (b) Hierarchical *F*-statistics. The levels of the hierarchy consist of: 1) populations, 2) genetic neighborhoods, 3) habitats, and 4) individuals. These statistics are used to calculate the variation in hierarchy components reported in Table 4b, following Weir (1996). The upper and lower boundaries given represent a 95% CI, generated by bootstrap analysis of the overall multi-locus statistics.

a. Coancestry coefficients

Locus	f	F	θ_S	θ_{SS}	θ_P
CT12	0.656	0.748	0.261	0.267	0.196
CT20	0.385	0.482	0.072	0.157	0.072
GA2	0.684	0.746	0.191	0.196	0.193
GA3	0.555	0.636	0.164	0.182	0.119
Overall	0.568	0.655	0.173	0.201	0.146
Upper bound	0.671	0.747	0.238	0.246	0.194
Lower bound	0.447	0.549	0.103	0.167	0.095

b. Hierarchical *F*-statistics.

Locus	Between individuals w/in habitats	Between habitats w/in location pairs	Between location pairs w/in populations	Between populations
CT12	0.656	0.008	0.081	0.196
CT20	0.386	0.092	0.000	0.072
GA2	0.684	0.006	-0.002	0.193
GA3	0.555	0.022	0.051	0.119
Overall	0.568	0.034	0.032	0.146
Upper bound	0.671	0.074	0.073	0.194
Lower bound	0.447	0.007	-0.002	0.095

Figures

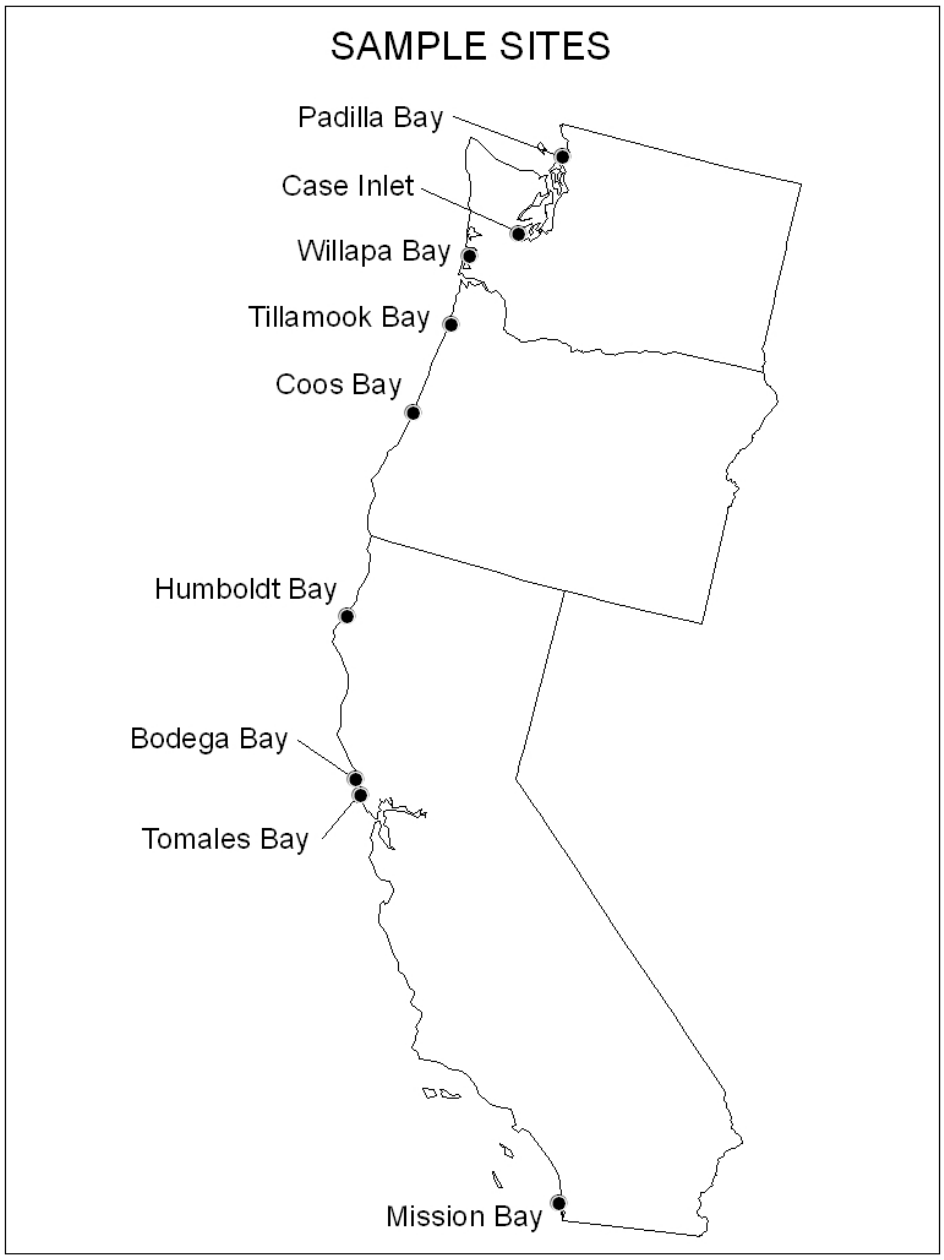
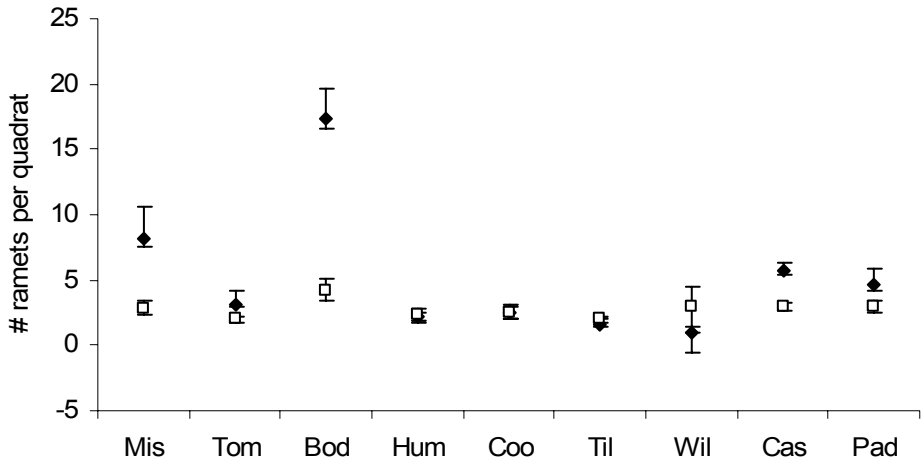
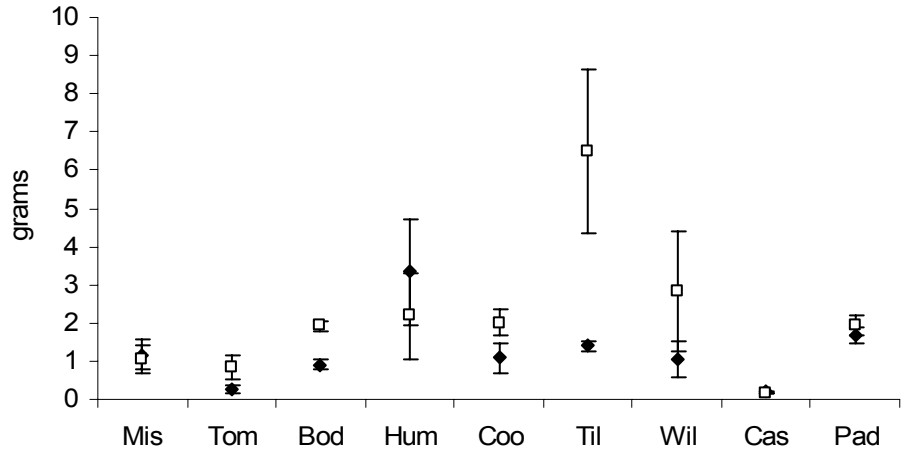


Figure 3.1. Names and locations of nine eastern Pacific *Z. marina* populations sampled are shown.

a. Mean vegetative density



b. Mean vegetative mass



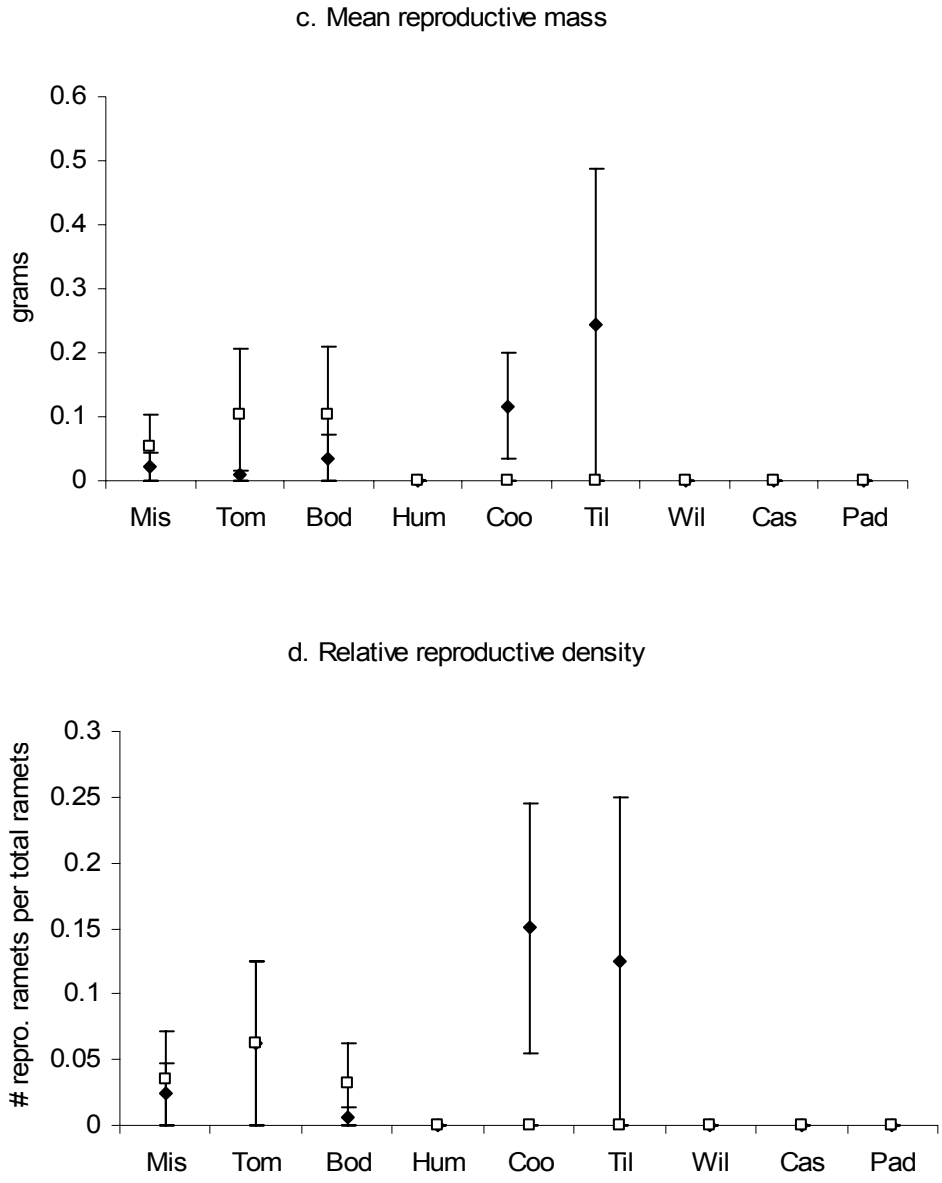


Figure 3.2a-d. Mean vegetative density, mean vegetative mass, mean reproductive mass, and relative reproductive density with standard error. Filled diamonds represent intertidal data and unfilled squares represent subtidal data. Populations are ordered along a latitudinal gradient, with the southernmost population (Mission Bay) at the left. Mean vegetative density and mean vegetative mass differ significantly by habitat ($p < 0.05$).

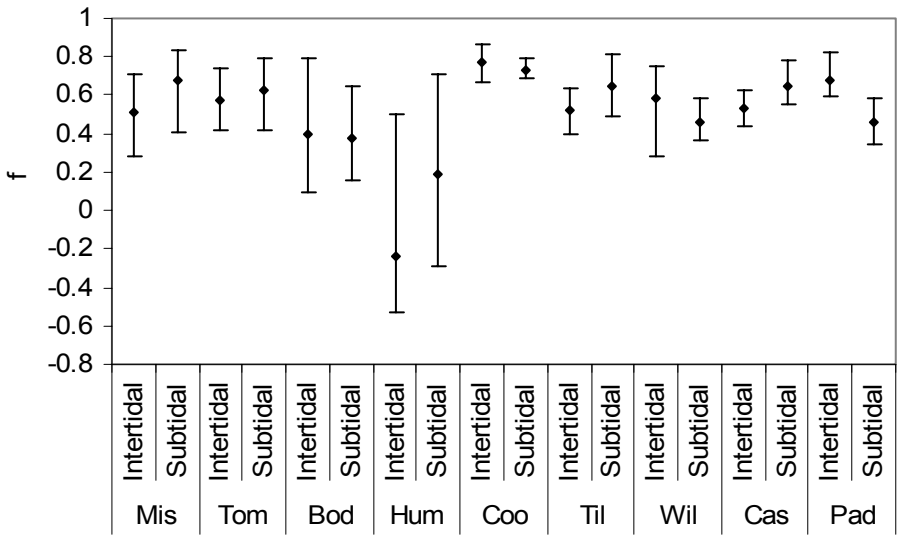


Figure 3.3. Inbreeding coefficient (f) is shown with a 95% confidence interval for each habitat and population. Populations are ordered along a latitudinal gradient, with the southernmost population (Mission Bay) at the left. Intervals that do not overlap differ, with $\alpha = 0.05$.

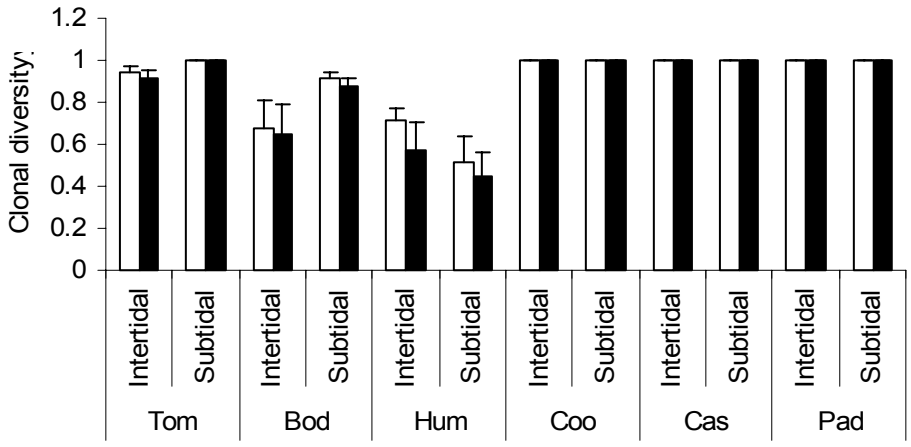


Figure 3.4. Mean clonal diversity of intertidal and subtidal habitats for each population sampled. White bars show Simpson's diversity index and black bars show Ellstrand and Roose's diversity index. Error bars indicate one standard error. The populations are arranged in a latitudinal gradient, with the southernmost population (Tomales Bay) located at the left side of the x-axis.

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